

Proteasome inhibitors as new anticancer drugs

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The targeted degradation of key regulatory proteins is an essential element of cell cycle control. The proteasome plays a central role in the degradation of such proteins and has therefore become an important therapeutic target for diseases involving cell proliferation, notably cancer. This review summarizes numerous studies demonstrating that proteasome inhibition induces apoptosis and sensitizes cancer cells to traditional tumoricidal agents both *in vitro* and *in vivo*. The potent and selective proteasome inhibitor, PS-341, is particularly promising from a therapeutic perspective, and it is the only such inhibitor that has progressed to clinical trials. Preliminary data indicate that the drug is well tolerated by patients with cancer, and further trials are underway to assess the safety and efficacy of proteasome inhibition in hematologic and solid tumors, both as a monotherapy and in combination with other chemotherapeutics. *Curr Opin Oncol* 2002, 14:628-634

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Abbreviations

MTD maximum tolerated dose
NFκB nuclear factor κB

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The 26S proteasome, a multicatalytic enzyme expressed in the nucleus and cytoplasm of all eukaryotic cells, has traditionally been viewed as a recycler of damaged or misfolded proteins, but it has become increasingly apparent over the last decade that the proteasome plays a vital role in degrading numerous proteins [1] that regulate the cell cycle and perhaps apoptosis. Proteasome substrates include transcription factors, signaling molecules, tumor suppressors, cell cycle regulators, and inhibitory molecules such as IκB and securin, among others [2]. Targeted protein degradation might also be required not only for the normal progression of the cell cycle but also for the accelerated and uncontrolled mitosis characteristic of cancer development and spread [3]. Inhibition of the proteasome may therefore arrest or retard cancer progression by interfering with the ordered degradation of cell cycle proteins [4••]. Indeed, the inhibition of proteasomal function results in programmed cell death (apoptosis) [5].

This review summarizes recent studies that have examined the use of proteasome inhibitors for the treatment of cancer.

Ubiquitin-proteasome pathway

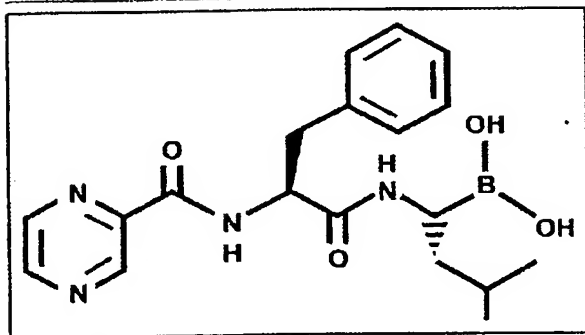
The targeting of proteins for proteasomal degradation is a highly regulated process. The primary mechanism by which proteins are directed to the proteasome involves the attachment of a polyubiquitin chain [6]. The first step of the ubiquitin-proteasome pathway is the covalent attachment of a single ubiquitin molecule to a free amino group on the target protein. The selectivity of protein targeting is largely conferred by the enzymes that perform this initial reaction [7•]. In subsequent reactions, further ubiquitin molecules are added to form the polyubiquitin chain. It is this chain that is recognized by the proteasome.

Structure of the proteasome

The 26S proteasome is a multiprotein complex consisting of a 20S core particle associated with one or two 19S regulatory particles (Fig. 1). The 19S subunit binds the polyubiquitin chain and cleaves it from the protein substrate. The substrate is then denatured, allowing access to the proteolytic core [2].

The 20S core particle is a barrel-shaped structure composed of four stacked rings in an α-β-β-α conformation. The outer α rings complex with the 19S regulatory particles, forming a narrow channel through which only de-

Figure 1. Schematic of 26S proteasome



The 26S proteasome is a 2000-kD multiprotein complex composed of a proteolytically active 20S core particle that is capped by one or two 19S regulatory particles. These regulatory particles recognize ubiquitinated proteins and control access to the proteolytic core. Published with permission from Millennium Pharmaceuticals, Cambridge, MA.

natured proteins may pass. The catalytic chamber is formed by the two inner β rings, each of which contains three active sites. These active sites account for the three major proteolytic activities of the proteasome: chymotrypsin-like, trypsin-like, and postglutamyl peptide hydrolase-like [8]. Proteins are degraded by the core particle in a progressive manner, generating peptides three to 25 residues in length [9].

Proteasome inhibitors

Numerous proteasome inhibitors have been described, many of which interfere directly with the proteolytic activity of the 20S core particle. These inhibitors bind either reversibly or irreversibly to active sites in the core particle, and they display varying levels of specificity for the 26S proteasome.

The synthetic peptide aldehydes, including Z-Leu-Leu-Leu-H (MG-132) and Z-Ile-Glu(OBzl)-Ala-Leu-H (PSI), were among the first proteasome inhibitors to be described [10]. These compounds reversibly inhibit the chymotrypsin-like activity of the proteasome such that protein degradation is restored upon their removal [4••]. However, they are also potent inhibitors of thiol proteases such as cathepsin B and the calpains, and they may display poor metabolic stability and bioavailability [10,11].

Lactacystin is a natural compound that hydrolyzes in aqueous solution to form *clasto*-lactacystin β -lactone, an inhibitor of all three proteolytic activities of the proteasome [12]. This inhibition is irreversible for trypsin-like and chymotrypsin-like activities [13]. Although β -lactone is more selective for the proteasome than the aforementioned peptide aldehydes [14], it does exhibit some activity against cathepsin A [15]. Furthermore, the inhibitor is unstable because β -lactone is itself hydrolyzed to form an inactive metabolite [12].

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Synthetic peptides possessing a carboxyl vinyl sulfone (VS) moiety are irreversible inhibitors of the proteasome. Peptides such as Z-Leu-Leu-Leu-VS (NLVS) are relatively easily synthesized and covalently block all three proteolytic activities of the proteasome [16]. However, vinyl sulfone-based inhibitors have limitations similar to those of the peptide aldehydes in that they bind and inhibit cathepsin S [16].

Natural epoxyketone-containing compounds such as epoxomicin and eponemycin are potent and irreversible proteasome inhibitors [17]. Epoxomicin primarily inhibits the chymotrypsin-like activity of the proteasome [17], whereas eponemycin and its synthetic analogue dihydro-eponemycin inhibit chymotrypsin-like and postglutamyl peptide hydrolase-like activity at comparable rates [18]. These agents are extremely selective proteasome inhibitors, with no activity against any other protease [17].

The TMC-95s are a class of cyclic peptides that inhibit the proteasome in a reversible manner. These compounds form hydrogen bonds with the protein backbone of the proteasome, sterically hindering access to the active sites [8]. The structure of the 20S proteasome-TMC-95A complex has recently been elucidated, and TMC-95A is seen as a model for the development of synthetic inhibitors suitable for clinical use [8].

From a clinical perspective, perhaps the most promising proteasome inhibitors are the peptide boronic acids. These compounds are as much as 1000-fold more potent than their aldehyde analogues and are extremely selective for the proteasome over common serine proteases [10]. The peptide boronic acids block the chymotrypsin-like activity of the proteasome in a reversible manner and dissociate from the proteasome at a slower rate than the peptide aldehydes. Within this new class of peptides, the dipeptidyl boronic acids offer the additional advantages of relatively low molecular weight and simplicity of synthesis [10].

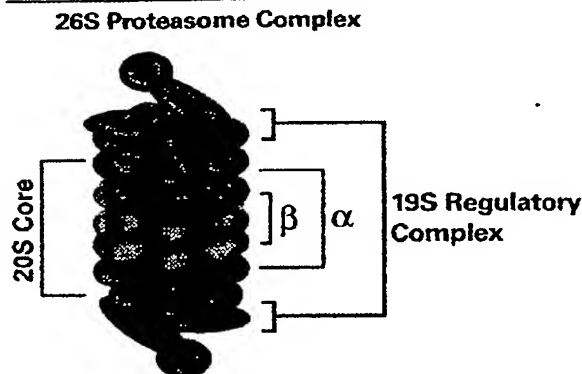
The compact, water-soluble dipeptidyl boronic acid PS-341 is of particular interest (Fig. 2). PS-341 is an extremely potent ($K_i = 0.6$ nM) and selective proteasome inhibitor. It binds to the proteasome with very high affinity and dissociates slowly, conferring stable but reversible proteasome inhibition [10]. These characteristics make PS-341 especially suitable for clinical use.

Activity of proteasome inhibitors *in vitro*

Proteolysis by the 26S proteasome is a fundamental metabolic process, and the complete blockade of proteasomal activity results in programmed cell death [5]. Importantly, however, antitumor activity can be achieved with less than maximal inhibition, and tumor cells seem to be more sensitive to proteasome inhibitors than are normal cells. For example, multiple myeloma cells [19]

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Figure 2. Structure of dipeptidyl boronic acid proteasome inhibitor PS-341



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and leukemic cells [20] are significantly more sensitive to the proapoptotic effects of proteasome inhibition than are CD34(+) bone marrow progenitor cells or lymphocytes from healthy people.

Actively dividing cells are also more sensitive to proteasome inhibition than are quiescent or differentiated cells. Thus, human leukemia cells that have been induced to differentiate are significantly less sensitive to the apoptotic effects of PSI than their rapidly proliferating precursors [21]. Similarly, quiescent endothelial cells are less sensitive to proteasome inhibitor-induced apoptosis than are dividing cells [22]. However, it is unlikely that whether the hypersensitivity of cancer cells to proteasome inhibition can be explained solely by their accelerated rate of division.

Proteasome inhibition also increases the sensitivity of cancer cells to traditional anticancer agents such as gemcitabine [23], cisplatin [20,24,25], paclitaxel [24], irinotecan (CPT-11) [26], and radiation [27,28]. Indeed, proteasome inhibition is sufficient to overcome the resistance of some cell types to conventional therapies [29,30]. For example, proteasome inhibition sensitizes murine lymphoma cells previously resistant to radiation-induced apoptosis, despite elevated expression of Bcl-2 [30]. Similarly, high doses of PS-341 induce apoptosis in MIA-PaCa-2 human pancreatic cancer cells, whereas low doses increase the cytotoxicity of gemcitabine [23].

The precise mechanisms by which proteasome inhibitors induce cell death are yet to be established. Although the stabilization of p21, p27, and p53 are common responses to proteasome inhibition [26,30,31,32,33], the involvement of these regulatory proteins in apoptosis varies between cell types. Thus, proteasome inhibitor-induced apoptosis is dependent upon p53 expression in some cell

lines [5,30] but not in others [19,26,29,32-34]. Current theories of apoptosis suggest that the initiation of programmed cell death is not mediated by any one protein, but rather by the ratio of antiapoptotic to proapoptotic proteins within a cell [35]. Proteasome inhibition may therefore induce apoptosis, or increase sensitivity to apoptosis, by disturbing this balance.

Effects on cell cycle-regulatory proteins and transcription factor nuclear factor κ B

The effects of proteasome inhibition on the stability of various cell cycle regulatory proteins, including cyclins (eg, cyclin B1), cyclin-dependent kinase inhibitors (eg, p21 and p27) [31], tumor suppressors (eg, p53), and the transcription factor nuclear factor κ B (NF κ B), are well established [36-38]. The last is of particular interest, because interference with the NF κ B transcriptional pathway appears to influence tumor cell survival [39].

Under normal circumstances, NF κ B is sequestered in the cytoplasm and rendered inactive by the inhibitor protein I κ B. In times of cell stress, however, I κ B is degraded by the proteasome, and NF κ B translocates to the nucleus. NF κ B promotes cell survival by initiating the transcription of genes encoding stress-response enzymes, cell-adhesion molecules, proinflammatory cytokines, and antiapoptotic proteins such as Bcl-2, cIAP1, and cIAP2 [40-43]. NF κ B is constitutively active in certain malignancies [44,45] and has been shown to promote tumor cell survival and reduce the effectiveness of anticancer therapy [37,46]. The constitutive activity of NF κ B may also be correlated with *in vitro* drug resistance [47].

Proteasome inhibition has been shown to block the chemotherapy-induced activation of NF κ B, resulting in enhanced chemosensitivity and increased apoptosis in cancer cells [26,48]. Similarly, inhibition of NF κ B activation increases radiation-induced apoptosis and enhances the radiosensitivity of colorectal cancer cells *in vitro* and *in vivo* [28].

Cytotoxic effects on tumor cell lines

Several proteasome inhibitors have demonstrated antitumor activity *in vitro*. For example, lactacystin and MG-132 potently induce apoptosis in B cells isolated from patients with B-cell chronic lymphocytic leukemia [49]. These agents also induce apoptosis in cultured p53-defective leukemic cells [34] and in several human malignant glioma lines [50]. Similarly, MG-132 has been shown to induce apoptosis in HD-My-Z Hodgkin cells and in p53-competent and p53-defective gastric cancer cells [32].

A standard National Cancer Institute screen of 60 cancer cell lines derived from multiple human tumors has shown PS-341 to have potent anticancer activity *in vitro* [33]. PS-341 was found to penetrate cancer cells and

inhibit the proteolysis of long-lived proteins, with 50% proteolytic inhibition observed at concentrations of approximately 0.1 μ M. Further, PS-341 potently inhibited the growth of these cells, with an average dose of only 7 nM required to achieve 50% growth inhibition. Importantly, a comparison of the cytotoxic profile of PS-341 with the historical results of 60,000 other compounds found it to be unique, with little similarity to other "standard" or investigational agents [33]. Additional studies have subsequently confirmed the cytotoxicity of PS-341 in human MCF-7 breast carcinoma cells [51] and BxPC3 pancreatic cancer cells [31•].

There are important indicators that these *in vitro* results will be relevant to tumor growth *in vivo*. PS-341 is as effective against multicellular spheroid cultures as against monolayer cultures and thus may show promising activity against solid tumors with low growth fractions [52]. Furthermore, PS-341 induces apoptosis in proliferating endothelial (human umbilical vein endothelial) cells [53] and prevents vascularization in an embryonic chick chorioallantoic membrane system [22], suggesting that proteasome inhibition may limit angiogenesis *in vivo*.

Activity of proteasome inhibitors in tumor-bearing animal models

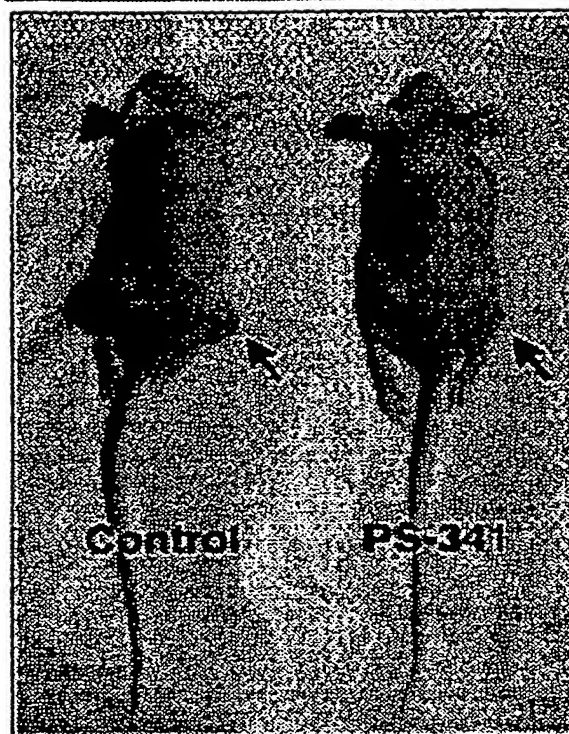
PS-341 is the only proteasome inhibitor to have been extensively studied in xenograft models. Preliminary data indicate that PS-341 has significant activity against human xenografts of multiple myeloma [54], mantle cell lymphoma-xenografted severe combined immunodeficiency mice [55], pancreatic cancer [23,31•] colon cancer [26•], prostate cancer [33], and squamous cell carcinoma [39•], with some animals exhibiting complete tumor regression.

In concurrence with *in vitro* studies, proteasome inhibition appears to increase the potency of traditional tumoricidal agents in an additive or synergistic manner in xenograft models. PS-341 dramatically inhibits tumor growth in murine xenograft models of human pancreatic cancer when used in combination with gemcitabine [23] or CPT-11 [31•] and human colon cancer when used in combination with CPT-11 therapy [26•]. PS-341 also significantly inhibits tumor growth in colon cancer-xenografted mice receiving ionizing radiation (Fig. 3) [28].

Similarly, proteasome blockade inhibits the growth of grafted murine prostate tumors treated with radiation [27] and grafted murine mammary tumors treated with cisplatin, cyclophosphamide, or radiation [51]. Moreover, PS-341 alone and in combination with other tumoricidal agents is highly effective against lung metastatic disease in mice [51].

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Figure 3. Proteasome inhibition increases potency of traditional tumoricidal agents



PS-341 treatment in combination with radiotherapy significantly reduces the growth of colorectal cancer xenografts in mice. The mouse on the left received a single injection of saline, whereas the mouse on the right received a single injection of PS-341 (1 mg/kg). Tumors on the right flank of both animals were then irradiated (6 Gy). The combination of PS-341 and radiation has a greater tumoricidal effect than either PS-341 or radiation alone. Published with permission [28].

Histologic analysis indicates that tumor growth inhibition in murine xenografts is accompanied by an increase in cancer cell apoptosis. Treatment with PS-341 alone is sufficient to induce apoptosis in human mantle cell lymphoma xenografts [55], whereas combined PS-341 and CPT-11 therapy yields highly apoptotic cells in mice bearing human colon [26•] or pancreatic tumors [31•]. Histologic analysis of blood vessel density also reveals that, in addition to its direct cytotoxic effects, PS-341 appears to have antiangiogenic activity in tumors [39•].

PS-341 acts in a dose-dependent manner in xenograft models and is well tolerated in doses as high as 0.5 or 1.0 mg/kg body weight [26,28,54]. Studies of drug distribution after intravenous injection indicate that, whereas most organs receive a similar amount of the drug, PS-341 is present at relatively low levels in the skin. Thus, subcutaneous tumors would have limited exposure to the drug, and the potency of PS-341 may be underestimated

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in this model system [33]. Proteasome inhibition was not detected in the central nervous system, eyes, or testes, suggesting that PS-341 does not readily cross tight endothelial cell junctions [33].

Studies are underway to investigate the properties of xenografted tumors grown from PS-341-resistant multiple myeloma cells [56].

Proteasome inhibitors as new cancer drugs in clinical trials

PS-341 is the only proteasome inhibitor to have progressed to clinical trials. More than 30 clinical trials sponsored by Millennium Pharmaceuticals, the Cancer Therapy Evaluation Program of the National Cancer Institute, or independent investigators are evaluating the use of PS-341 in patients with hematologic malignancies or solid tumors. Approximately 200 patients have thus far received the drug in phase I studies, with an additional 256 in phase II myeloma trials and numerous others in the Cancer Therapy Evaluation Program.

Because PS-341 is cleared rapidly from the plasma compartment, traditional pharmacokinetic parameters have not proven useful for dose optimization in phase I trials. A novel bioassay has therefore been used as a guide to dose escalation in phase I studies. This assay rapidly and reliably measures residual proteasome activity in whole blood or white blood cells, and it may also be used in tissue biopsies to quantify the effect of the drug in solid tumors. Bioassay testing in volunteers indicated minimal intersubject and intrasubject variation in proteasome activity. In rats, bioassay testing and whole-body autoradiography after administration of (^{14}C)PS-341 indicate that most organs receive approximately the same amount of the drug [33].

The effects of PS-341 are clearly dose-dependent. Toxicology studies in rodents and primates show that the primary adverse events associated with PS-341 treatment are anorexia, vomiting, and diarrhea. These gastrointestinal adverse events are dose-related, with PS-341 generally well tolerated to as much as 80% proteasome blockade [33].

A single maximum tolerated dose (MTD) has not been established in clinical trials. Indeed, preliminary data indicate that MTD may vary by tumor type and treatment schedule. Phase I trials in solid tumors are assessing escalating doses of PS-341 in combination with common chemotherapeutic agents such as gemcitabine, irinotecan, and docetaxel, among others. Phase II trials in hematologic malignancies are currently exploring doses of 1.3 mg/m² twice weekly for 2 weeks, with 1 week of rest. Preliminary analysis of one of these trials [57] revealed that, of 78 patients analyzed, approximately 7% of patients responded (complete, partial, or minor), including

20% of patients with more than 90% reduction in paraprotein, and 30% had stable disease. A randomized multicenter international phase III trial comparing PS-341 with dexamethasone in patients with relapsed disease began in the first half of 2002.

Based on the results in xenograft experiments using combination therapy, PS-341 is being combined with numerous standard agents in dose-ranging phase I trials. To date, the trials indicate that the combination of PS-341 and gemcitabine or irinotecan has manageable toxicities [58,59]. Based on the results of the PS-341 and gemcitabine phase I trial in advanced solid tumors, the recommended regimen to be assessed in phase II trials of pretreated patients is 1.0 mg/m² PS-341 and 1000 mg/m² gemcitabine [58]. This trial continues to enroll chemotherapy-naïve patients to determine the MTD of this combination in patients who have not received previous chemotherapy. MTD in the PS-341 and irinotecan trial has not yet been reached [59]. Future phase II studies are planned.

Conclusions

During the last year, numerous preclinical studies have demonstrated that proteasome inhibition has a direct antitumor effect and sensitizes cancer cells to anticancer agents in current use. The mechanism by which proteasome inhibition induces apoptosis is under investigation, and these studies provide a rationale for further examination of proteasome inhibitors as potential therapeutics. Preliminary clinical trials in patients with cancer indicate that PS-341 is well tolerated at doses significantly inhibiting proteasome activity, and that further studies are justified.

In addition to its antitumor effects, proteasome inhibition may prove effective in the treatment of conditions involving inflammation [60], because many of the molecules that mediate inflammation are affected by proteasomal degradation. The proteasome is therefore an attractive target for drug development, and PS-341 is the first in a potentially promising therapeutic class.

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